CELLULAR IMMUNOTHERAPY

BACKGROUND OF THE INVENTION

The present invention relates to immune mediated diseases as typified by immunosuppressive virus infections exemplified by immunosuppressive virus infection (both human, *e.g.*, HIV, and animal, *e.g.*, FIV) and more particularly to the adoptive cellular therapy of such virus infections. Much of the discussion will focus on HIV, although such discussion is by way of illustration and not by way of limitation.

Antiviral therapy in persons with AIDS or advanced HIV infection has failed to cure the infection or arrest the deterioration of the immune system. One possible approach to treating the infection and reconstituting or restoring the immune system is to enhance the patient's cellular antiviral capacity. Certain cytokines are capable of inducing the replication of HIV; therefore, selective blocking of cytokines is an important strategy in the treatment of HIV disease. However, cytokines also are important for the expression and regulation of normal immune function. Defects in the production of, *e.g.*, interleukin-2 (IL-2) and interferon (IFN) have been reported in HIV infection, and the administration of cytokines in order to partially reconstitute or stimulate the defective cellular immune system in HIV disease is a strategy that is being pursued. Fauci, "Multifactorial nature of human immunodeficiency virus disease: implications for therapy", *Science*, 262:1011-1018, 1993. A number of chemical immune enhancers have also been proposed for the treatment of HIV-infected individuals, with clinical trials generally showing variable results. Turowsky, *et. al.*, "Clinical application of chemical immunomodulators in cancer and AIDS", *Cancer Invest*, 1994, vol. 12, 620-643 (1994).

The most direct and potentially most effective form of immunologic reconstitution is the replacement of cellular elements of the immune system, *i.e.*, adoptive cellular therapy. Based on studies showing that $CD8^+$ cytolytic T cells (CTL) from HIV-infected patients have anti-HIV activities, most efforts have focused on $CD8^+$ cells. Ho *et al.* conducted a phase I study to determine the safety and feasibility of infusing *in vitro* purified, activated, and expanded $CD8^+$ cells in seven patients with AIDS-related complex or AIDS. Ho, *et al.*, "A phase I study of adoptive transfer of autologous $CD8^+$ T lymphocytes in patients with acquired immunodeficiency syndrome (AIDS)-related complex or AIDS", *Blood*, 81:2093-2101, 1993. Autologous $CD8^+$ cells were first selectively isolated in monoclonal antibody-coated flasks from peripheral blood mononuclear cells (PBMC) recovered by leukopheresis. They were then cultured and expanded with phytohemagglutinin and IL-2 before infusion. Five cycles of isolations and infusions of increasing numbers of $CD8^+$ T

cells were achieved in five of seven subjects. Five cycles could not be completed in two subjects with AIDS whose $CD4^+$ cell counts were < 48/μL. Infusions of $CD8^+$ cells alone were well tolerated. Four patients received IL-2 by continuous infusion for 5 days with their final cycle of $CD8^+$ cells. All developed reversible adverse effects attributable to IL-2. After infusion, ^{111}In-labeled $CD8^+$ cells quickly accumulated in the lungs, with less than 10% of the labeled cells remaining in the circulation. After 24 hours, labeled $CD8^+$ cells were reduced in the lungs, but increased and persisted in liver, spleen, and bone marrow. Four of five patients who were treated with multiple infusions as $CD8^+$ cells remained clinically stable, but a fifth developed *Pneumocystis carinii pneu*monia. Other attempts to reconstitute cellular elements under investigation include the transfusion of *ex vivo* IL-2 expanded HIV-specific $CD8^+$ T cell clones, bone marrow transplantation, and thymic transplantation. Riddell, *et al.*, "$CD8^+$ cytotoxic T cell therapy of cytomegalovirus and HIV infection", *Curr Opin Immunol*, 5:484-491, 1993; and Fauci, *et al.*, *supra.*

Results are too preliminary for conclusions to be reached concerning the feasibility and long-term benefit of adoptive cellular therapy programs focusing on $CD8^+$ CTL. However, there are a number of potential limitations: *(i)* Normally $CD8^+$ cells do not make enough IL-2 to support their own expansion and are dependent on IL-2 and possibly other cytokines from $CD4^+$ cells for "help". In the absence of T_H activity, HIV-specific CTLs would not be expected to expand and eliminate HIV *in vivo*. *(ii)* Virus-specific $CD8^+$ CTLs have been demonstrated to cause immunopathology if a significant reservoir of actively infected cells is established in a vital organ as the response is elicited. HIV-specific CTLs have been implicated in the pathogenesis of AIDS dementia and lymphocytic alveolitis (Riddell, *supra*). *(iii)* Peripheral blood-derived $CD8^+$ do not appear to traffick to major sites of HIV activity, *e.g.*, lymph nodes (Ho, *et al.*, *supra*). *(iv)* There is evidence that HIV reactive $CD8^+$ cells may contribute to the immunodeficiency by lysing HIV-infected CD4 cells. Grant, *et. al.*, "Lysis of CD4+ lymphocytes by non-HLA-restricted cytotoxic T lymphocytes from HIV-infected individuals", *Clin Exp Immunol*, 93:356-362, 1993. *(v)* A major role in the progression of HIV infection is played by the HIV-mediated destruction of the microenvironment of the lymphoid organs. It is unclear whether the severely damaged immune systems of patients with advanced HIV disease would be capable of spontaneous regeneration even if virus replication could be completely inhibited by totally effective $CD8^+$ CTLs. In fact, there is suggestive evidence that spontaneous regeneration would not occur under these circumstances (Fauci, *supra*).

The fundamental distinguishing feature between immunotherapy of HIV infection and that for other persistent viruses is that HIV undermines the ability of the host to appropriately respond to efforts to augment of elicit potentially protective host immune responses by inducing dysfunction and depletion of $CD4^+$ T_H cells. The

presentation of an antigen (Ag) to $CD4^+$ T_H cells and the generation of T_H activity are the central processes in any specific cellular immune response, such as the specific response to a HIV, upon which all the other elements depend. Murine $CD4^+$ T_H cells comprise at least three subsets (Powrie, *et al.*, "Cytokine regulation of T-cell function: potential for therapeutic intervention", *Immunol Today*, 14:270-274, 1993): The T_H1 subset, which secretes IL-2 and IFN, but not IL-4, appears to be responsible for delayed type hypersensitivity (DTH) responses. T_H2 cells, which secrete IL-4 and IL-5, but not IL-2, provide B-cell help. T_H0 cells produce IL-2, IFN, IL-4, and IL-5. Several groups have now isolated human clones similar to the mouse T_H1, T_H2, and T_H0 phenotype. The factors influencing T_H differentiation are not completely understood; current evidence suggests the following: T_H1 differentiation occurs in response to intracellular viruses and bacteria and is promoted by IFN, IFNa, and IL-12. T_H2 differentiation occurs in response to soluble allergens and some helminth components and is promoted by IL-4 and, in some studies, transforming growth factor (TGF) ß. In addition to the depletion of the T_H arm of the immune response of HIV-infected individuals, recent reports suggest that there is a progressive imbalance immune systems with a selective defect in T_H1 responses and a predominance of T_H2 responses. Correcting this imbalance by the administration of T_H1-type cytokines such as IL-2 or IL-12 is under investigation.

It is now evident that the initial host cellular immune response to HIV reduces the level of HIV in the peripheral blood. In the early, "immune activation" stage of infection, HIV is harbored in lymphatic tissues. Lymph nodes are characterized by germinal center hyperplasia and a $CD4^+$ cell pleocytosis that includes a substantial number of IL-2 and IFN secreting cells suggesting that a T_H1 differentiation has occurred. Emille, *et. al.*, "Production of interleukins in human immunodeficiency virus-1-replicating lymph nodes", *J Clin Invest*, 86:148-159, 1990. Recently, the use of sensitive molecular techniques combined with careful histopathologic examination has indicated that in the early stages HIV exists primarily as extracellular virus trapped in the follicular dendritic cell network of the germinal center. Intracellular virus also is detectable but usually in a latent form. It is postulated that the activation of $CD4^+$ T cells by antigen presenting cells (APC), which normally occurs in the follicular center, renders uninfected cells susceptible to infection by the locally retained virus and results in the reactivation of HIV in latently infected cell, thus contributing to the gradual depletion of $CD4^+$ T_H cells observed during the course of HIV infection.

Nonetheless, it does appear that early in the course of HIV infection an appropriate and potentially effective immune response is generated in lymph nodes. We are proposing to examine the possibility that cells that can provide appropriate T_H function can be isolated and expanded *ex vivo* from the lymph nodes infected with HIV

and that adoptive cellular therapy using the expanded lymph node lymphocytes (LNL) will generate antiviral immunity *in vivo*. It may appear counter-intuitive to utilize a major reservoir of HIV, *i.e.*, lymph nodes, and the central target of HIV infection, *i.e.*, activated $CD4^+$ cells, in the adoptive cellular therapy of HIV infection. Based on the central role of lymph nodes and $CD4^+$ cells in immune response, the possibility does exist that under certain circumstances, and perhaps in association with the administration of effective antiretroviral agents, stimulation of the immune system by $CD4^+$. LNL secreting appropriate cytokines might have a beneficial effect in reconstituting immune competence in HIV infected individuals. It should be noted that although $CD8^+$ CTL suppress the replication of HIV in PBMC of patients with AIDS, Walker *et al.* discovered that the virus infected cells were not killed during the process, but rather that HIV replication was inhibited by a diffusible cytokine elaborated by $CD8^+$ cells. Walker *et al.*, "A diffusible lymphokine produced by CD8+ T lymphocytes suppresses HIV replication", *Immunology*, 66:628, 1989. Furthermore, it has been demonstrated that collaboration between $CD4^+$ T cells and B cells and the factors that these cells produce are essential for the normal development of the lymphoid microenvironment (Fauci, *supra*). Lymph nodes are also attractive as a source of cells not only because they contain all the critical elements to develop an immune response, *e.g.*, APCs, but also because the yield of immunologically active cells is orders of magnitude greater than that from the peripheral blood. The locomotor capability of LNL, and, thus, the ability to traffick to lymph nodes, also may be greater than that of PBL. Triozzi, "Identification and activation of tumor-reactive cells for adoptive immunotherapy", *Stem Cells*, 11:204-211, 1993.

BROAD STATEMENT OF THE INVENTION

Broadly, disclosed is a novel approach to the adoptive cellular therapy of immune mediated diseases exemplified by HIV infection which therapy exploits the potentially effective cellular immune response that is initially generated in HIV-infected individuals. One aspect is a method for preparing cells having an immunorestorative effect on patients afflicted with human immunodeficiency virus (HIV), which includes subjecting cytokine-producing cells derived from lymph nodes excised from patients infected with HIV to mitogenic stimulation in serum-free media for their expansion. The resulting therapeutic agent is effective in inducing an immunorestorative effect on patients afflicted with human immunodeficiency virus (HIV) and includes in a pharmaceutically-acceptable carrier cytokine-producing cells in an amount effective for HIV reduction, the cytokine-producing cells having been produced by the step of subjecting cytokine-producing cells derived from lymph nodes excised from patients infected with HIV to mitogenic stimulation in serum-free media for their expansion. As another aspect of the present invention, disclosed is a method for inducing an

immunorestorative effect on patients afflicted with human immunodeficiency virus (HIV) which includes administering to the patient an effective amount of the therapeutic agent disclosed herein.

The invention apparently is capable of inhibiting replication of HIV as determined by the viral load reductions exhibited by patients administered the therapeutic agent of the present invention. Thus, another aspect of the present invention is a therapeutic agent effective in reducing the viral load in patients afflicted with human immunodeficiency virus (HIV) and includes in a pharmaceutically-acceptable carrier cytokine-producing cells in an amount effective for HIV reduction, the cytokine-producing cells having been produced by the step of subjecting cytokine-producing cells derived from lymph nodes excised from patients infected with HIV to mitogenic stimulation in serum-free media for their expansion. As another aspect of the present invention, disclosed is a method for inducing a viral load reduction in patients afflicted with human immunodeficiency virus (HIV) which includes administering to the patient an effective amount of the therapeutic agent disclosed herein.

Advantages of the present invention include a culture procedure that is easy, expedient, and reproducible. Another advantage is a therapeutic agent that is negative for HIV; yet, is effective in inhibiting the replication of HIV. A further advantage is that the capacity of the therapeutic agent to secrete cytokine may obviate the need for exogenous systemic cytokines to maintain their activity. A further advantage is that total lack of adverse side effects when using the novel therapeutic agent. A yet further advantage is the lack of adverse reactions between the novel therapeutic agent and other drugs taken by the patient. These and other advantages will be readily apparent to those skilled in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 graphically depicts the influence of anti-CD3 MAb concentration on lymph node cell expansion. Cells were cultured for 10 days with a range of concentrations of anti-CD2 MAb (OKT3) and constant concentrations of IL-2. Data represent range and mean values for 5 different donors with peripheral blood CD4 counts from 46 to 210/mm^3.

Figs. 2 and 3 graphically depict expansion of lymph node and peripheral blood CD4 and CD8 cells from 2 patients. The peripheral blood CD4 count of the patient depicted in A was 23 and that of the patient depicted in B was 125/mm^3.

Fig. 4 graphically depicts supernatant cytokine levels (A) and cytokine production in response to immobilized anti-CD3 MAb (B). Data represent mean values cells stimulated with 5 (solid), 20 (cross-hatched), or 100 (dotted) ng/ml anti-CD3 MAb (n = 3).

Fig. 5 graphically depicts cytokine production in response to immobilized anti-

CD3 MAb of cells cultured for 4, 8, 10, and 12 days. Data represent mean values (n = 3).

Fig. 6 depicts HIV-1 mRNA expression of lymph node cells during *ex vivo* expansion with anti-CD3/IL-2, IL-2 alone, or with no additions (NA).

Fig. 7 graphically depicts the effect of culture supernatants generated with either 5, 20, or 100 ng/ml anti-CD3 MAb on HIV-1 replication added at an 80% concentration. Data are expressed as % suppression of p24 production.

Fig. 8 graphically depicts proliferative responses of expanded cells to oligopeptides corresponding to HIV-1 envelope sequence.

The drawings will be described in more detail below.

DETAILED DESCRIPTION OF THE INVENTION

The infusion of immunologically active cells is an attractive approach to AIDS. Cellular immunotherapy has been extensively studied in patients with cancer and has resulted in reported sustained complete responses and reported improved survival and quality of life (Rosenberg, S.A., "Adoptive Cellular Therapy: Clinical Applications", DeVita VT, Hellman, S., Rosenberg, S.A. (eds). *Biologic Therapy of Cancer*, Philadelphia: J.B. Lippincott, 1991, p. 214; Osband, *et al.*, "Effect of Autolymphocyte Therapy on Survival and Quality of Life in Patients with Metastatic Renal-Cell Carcinoma", *Lancet* 335:994-998,1990). Antiviral responses to, *e.g.*, cytomegalovirus, have also been effected in clinical trials (Riddell, *et al.*, "Restoration of Viral Immunity in Immunodeficient Humans by Adoptive Transfer of T-cell Clones", *Science* 257:238-240, 1992). A limited number of early phase clinical trials have been conducted in patients with HIV-1 infection. Ho, *et al.*, "A Phase I Study of Adoptive Transfer of Autologous $CD8^+$ T Lymphocytes in Patients with Acquired Immunodeficiency Syndrome (AIDS)-Related Complex or AIDS", *Blood* 81:2093-2101, 1993, infused purified autologous, peripheral blood $CD8^+$ CTL, expanded *ex vivo* with IL-2 and phytohemagglutinin (PHA). Four of five patients treated remained clinically stable, but a fifth developed *Pneumocystis carinii pneumonia*. Increases in circulating HIV-1-specific CTL activity were reported Torpey, *et al.*, "Effects of Adoptive Immunotherapy with Autologous $CD8^+$ T Lymphocytes on Immunologic Parameters: Lymphocyte Subsets and Cytotoxic Activity", *Clin. Immunol. Immunopath.* 68:263-272, 1993. Klimas, *et al.*, "Clinical and Immunological Changes in AIDS Patients Following Adoptive Therapy with Activated Autologous CD8 T Cells and Interleukin-2 Infusion, *AIDS* 8:1073-1081, 1994 infused six patients with IL-2/PHA-expanded, peripheral-blood, $CD8^+$ cells along with IL-2. Clinical improvement in lymphadenopathy, hairy leukoplakia, and Kaposi's sarcoma was observed. Carter, *et al.*, "Expansion of Syngeneic T Lymphocytes for Adoptive Immunotherapy in Twins Discordant for HIV Infection", *Blood* 82:416a, 1993, treated twelve HIV-1 patients

with repeated infusion of peripheral-blood lymphocytes from their HIV-1$^-$ twin that had been expanded *ex vivo* using anti-CD3 MAb and IL-2. Increases in CD4 counts were observed in patients with initial counts less than 200/mm^3; after a transient increase, p24 levels were also observed to decrease slightly. Other approaches under investigation include HIV-1-specific CTL generated by culturing peripheral blood lymphocytes with HIV-1 peptides, autologous cloned HIV-1-specific CTL transduced with a fusion marker/suicide gene, and CD8$^+$ cells transduced with genes coding for chimeric T-cell receptors that bind HIV-1-infected cells (reviewed by Bridges, *et al.*, "Gene Therapy and Immune Restoration for HIV Disease", *Lancet* 345:427-432, 1995).

Dysregulation of the cytokine system plays a central role in the progression of HIV-1 infection and related immunosuppression. The inventive approach to the cellular therapy of AIDS relies on lymph-node cell infusions as a means of modulating the cytokine system, rather than as a means of administrating HIV-1-specific CTL. The results reported herein indicate that significant numbers of cytokine-releasing cells with anti-HIV-1 activity can be expanded in short-term cultures from the lymph nodes of HIV-1 infected individuals, even from individuals with advanced disease. A variety of cytokines that could inhibit HIV-1 replication are potentially produced by the expanded cells, including: IL-8, IL-10, TNFa, and TGFb (Mackewicz, *et al.*, "CD8$^+$ Cell and Anti-HIV Activity: Nonlytic Suppression of Virus Replication" *AIDS Res. Hum. Retrovirus* 8:629-640, 1992). Supernatants from the expanded cells were capable of suppressing HIV-1 mRNA expression in cells initially cultured in IL-2 alone. Thus, it also is possible that CD8 cell anti-viral factor, as described by Levy and coworkers (Mackewicz, *et al.*, (*supra*); Mackewicz, *et al.*, "Effect of Cytokines on HIV Replication in CDR$^+$ Lymphocytes: Lack of Identity with CD8$^+$ Cell Antiviral Factor", *Cell Immunol.* 153:329-343, 1994; and Mackewicz, *et al.*, "CD8$^+$ T Cells Suppress Human Immunodeficiency Virus Replication by Inhibiting Viral Transcription", *Proc. Natl. Acad. Sci. USA* 92:2308-2312, 1995), is present. The cytokines produced would also be predicted to have a variety of immunorestorative effects.

The heterogeneity of HIV-1 makes is difficult to select cells reactive with all strains present at the time of re-infusion. Walker *et al.*, "Long-Term Culture and Fine Specificity of Human Cytotoxic T-Lymphocyte Clones Reactive with Human Immunodeficiency Virus Type 1", *Proc. Natl. Acad. Sci. USA* 86:9514-9518, 1989, have shown that MHC class I-restricted CTL specific for HIV-1 reverse transcriptase can be expanded from HIV-1-infected individuals. These CTL recognized multiple epitopes in conjunction with different host MHC molecules. There was a lack of cross-reactivity when CTL from other subjects were evaluated, suggesting that the immunogenic epitopes were different in different individuals. To generate cells with a

wide spectrum of HIV-1 reactivities, the invention used HIV-1-infected lymph nodes as a source of cells, rather than peripheral blood (PB). The relative frequencies of CTL specific for HIV-1 proteins from lymphoid organs differ at least one log from that of peripheral blood (Hadida, *et al.*, "CTLs from Lymphoid Organs Recognize an Optimal HLA-A2-Restricted and HLA-B52-Restricted Nonpeptide and Several Epitopes in the C-Terminal Region of HIV-1", *Nef. J. Immunol.* 154:4174-4186, 1995). The high levels of local viral replication and production of viral antigens together with presence of "professional" APCs, such as macrophages and dendritic cells, should maintain the activation and differentiation of a large array of CTL clones within lymph nodes. It also would seem likely that polyclonal response against HIV-1 are first elicited in lymphoid tissues before being disseminated in the peripheral blood.

The present invention uses the capacity of anti-CD3 MAb to mimic the normal pathways of T-cell activation and the capacity of IL-2 to expand multiple T-cell subpopulations from lymph nodes. HIV-1-specific T-cell lines can be expanded by nonspecific stimulation with anti-CD3 MAb and IL-2 without the need for re-exposure to viral antigen (Walker, *et al.*, (*supra*); Johnson, *et al.*, "HIV-1 Gag Specific Cytotoxic T Lymphocyte Recognize Multiple Highly Conserved Epitopes. Fine Specificity of the Gag-Specific Response Defined by Using Unstimulated Peripheral Blood Mononuclear Cells and Cloned Effector Cells", *J. Immunol.* 147:1512-1521, 1991; Littaua, *et al.*, "An HLA C Restricted $CD8^+$ Cytotoxic T Lymphocyte Clone Recognizes a Highly Conserved Epitope on Human Immunodeficiency Virus Type 1 Gag", *J. Virol.* 65: 4051-4056, 1991; Lieberman, *et al.*, "Cytotoxic T Lymphocytes from HIV-1 Seropositive Individuals Recognize Immunodominant Epitopes in GP160 and Reverse Transcriptase", *J. Immunol.* 148:2738-2747, 1992; and Buseyne, *et al.*, "Gag-Specific Cytotoxic T Lymphocytes from Human Immunodeficiency Virus Type 1-Infected Individuals: Gag Epitopes are Clustered in three Regions of the $p21^{gag}$ Protein", *J. Virol.* 67:694-702, 1993). It is difficult to assess the spectrum of HIV-1 reactivities *in vitro* of cells cultured in the presence of polyclonal activators, such as anti-CD3 MAb and IL-2, particularly in short-term bulk cultures. A proliferative response to a variety of HIV-1 antigens was observed. Although the expanded cells did proliferate in response to HIV-1 antigens, specific CTL activity against these targets was not demonstrated. HIV-1 specific CTL and CTL precursors likely were present in culture and may have played a role in the anti-HIV-1 activity. The inventive expansion regimen, however, was not designed to maximize cytolytic activity, and cells were exposed to decreasing concentrations of IL-2 with the intent of reducing their dependency on IL-2. Although the possibility was not examined, cells with reactivities against other pathogens to complicate HIV-1 infection also may have been expanded.

Culture conditions were optimized to maintain the viability of the APCs present in the lymph nodes, so that at least theoretically, any HIV-1-associated antigens

released *in vitro* could be presented by the APCs. Cells were cultured in serum-free conditions using a media designed to maintain the viability of macrophages and dendritic cells, and not lymphocytes. Cells expanded in serum-free conditions are potentially less toxic: the only significant toxicity observed in the study of Carter, *et al.*, (*supra*), was hypersensitivity to the bovine serum proteins that were adherent to the expanded lymphocytes. The concentration of anti-CD3 MAb influenced cell expansion. Moran, *et al.*, "Regulation of HIV Production by Blood Mononuclear Cells from HIV-infected Donors: I. Lack of Correlation between HIV-1 Production and T Cell Activation", *AIDS Res. Human Retroviruses* 9:455-464, 1993, have shown that although soluble anti-CD3 is a stimulus for HIV-1 production, immobilized anti-CD3 stimulation produces a factor that decreases HIV-1 replication. Both T cells and monocytes were found to be required for soluble anti-CD3 to induce high levels of HIV-1 production. A concentration of anti-CD3 MAb was used to elicit the effect of immobilized anti-CD3, by associating with the APCs present, and also elicit the effect of soluble anti-CD3 MAb and the possibility of *in vitro* immunization. The concentration of anti-CD3 MAb is 0.5% of that used by Moran, *et al.* (*supra*) to induce the effects of soluble anti-CD3. The transient increase in HIV-1 mRNA observed would be a predicted consequence of exposure to soluble anti-CD3 (Moran, *et al.*, *supra*). The presence of TNFa and IL-6 in the culture supernatants would be a predicted effect of immobilized anti-CD3 MAb as would the presence of soluble factors that inhibit HIV-1 (Moran, *et al.*, *supra*; Mackewicz, *et al.*, 1994, *supra*). Culture interval also influenced cytokine production; the greatest amounts of cytokines were produced by cells cultured for 8 to 10 days.

The expanded cells secreted both T_H1, *e.g.*, IFNg, and T_H2, *e.g.*, IL-5, cytokines, a result that would not be unexpected in light of the mixed population of cells present. Most of the cells expanding were $CD8^+$, as one of the unique features of lymph nodes during HIV-1 infection is the accumulation of large numbers of $CD8^+$ cells (Emilie, *et al.*, "Production of Interleukins in Human Immunodeficiency Virus-1-Replicating Lymph Nodes", *J. Clin. Invest.* 86:148-159, 1990). $CD8^+$ cells are the major producers of IFNg, a T_H1-type cytokine in HIV-1-infected lymph nodes; they also produce IL-4, IL-5, and IL-10, T_H2-type cytokines (Emilie, *et al.*, *supra*; Manetti, *et al.*, "CD30 Expression by $CD8^+$ T Cells Producing Type 2 Helper Cytokines. Evidence for Large Numbers of $CD8^+CD30^+$ T Cell Clones in Human Immunodeficiency Virus Infection", *J. Exp. Med.* 180:2407-2411, 1994; Graziosi, *et al.*, "Lack of Evidence of the Dichotomy of T_H1 and T_H2 Predominance in HIV-Infected Individuals", *Science* 265:248-252, 1994). The $CD8^+$ cells that expanded were $CD11b^-$, which is the predominant phenotype of anti-HIV-1 CTL and $CD8^+$ cells that suppress HIV-1 replication non-lytically (Mackewicz, *et al.*, 1992, *supra*). Although expansion rates varied, the cytokine profiles of cells expanded from

individuals with CD4 counts <100 and those with CD4 counts >100 were comparable. These results are consistent with those of Graziosi, *et al.* (*supra*), who found no correlation between the clinical stage of HIV-1-infected subjects as defined by CD4 counts and either the constitutive expression or that induced with immobilized anti-CD3-MAb-induced of T_H1 and T_H2 cytokines from lymph node cells in longitudinal and/or cross-sectional studies.

The expansion rates of CD4$^+$ cells present were comparable to the CD8$^+$ cells. In several model systems, the adoptive transfer of a mixed population of CD4$^+$ and CD8$^+$ cells has been more effective than purified CD8$^+$ cells, even when CD8$^+$ cells are central to desired response (Byrne, *et al.*, "Biology of Cloned Cytotoxic T Lymphocytes Specific for Lymphocyte choriomeningitis Virus: Clearance of Virus *in vivo*", *J. Virol.* 51:682-686, 1984; Larsen, *et al.*, "Role of T-Lymphocyte Subsets in Recovery from Herpes Simplex Virus Infection", *J. Vriol.* 50:56-59, 1984; Lukacher, *et al.*, "*In Vivo* Effector Function of Influenza Virus-Specific T Lymphocyte Clones is Highly Specific", *J. Exp. Med.* 160:814-823, 1984). Although the infusion of CD4$^+$ cells, activated CDF$^+$ in particular, are the principal target for HIV-1 and critical to the progression of the infection, there are theoretical advantages to infusing CD4$^+$ cells--activated CD4$^+$ in particular--are the principal target for HIV-1 and critical to the progression of the infection. There are theoretical advantages to infusing CD4$^+$ cells along with CD8$^+$ cells. CD8$^+$ cells normally do not make enough IL-2 to support their own expansion and are dependent on IL-2, and possibly other cytokines from CD4$^+$ cells for "help". In the absence of T_H activity, an infusion of HIV-1-specific CTL would not be expected to expand *in vivo*. There also is evidence, at least in the case of influenza infections, that *ex vivo* expanded CD4$^+$ cells can mediate antiviral effects directly (Scherle, *et al.*, "Functional Analyses of Influenza-Specific Helper T Cell Clones *In Vivo*: T Cells Specific for Internal Viral Protein Provide Cognate Help for B Cell Responses to Hemagglutinin", *J. Exp. Med.* 164:1114-1121, 1986). There may be other advantages of using a mixed population of cells. Antibodies have been able to protect against experimental retroviral infections under some circumstances (Vaslin, *et al.*, "Induction of Humoral and Cellular Immunity to Simian Immunodeficiency Virus: What are the Requirements for Protection", *Vaccine* 12:1132-1140, 1994); correlative evidence suggests that some antibody may be associated with protection against progress of HIV-1 infection (Salk, "Prospects for the Control of AIDS by Immunizing Seropositive Individuals", *Nature* 327:473-476, 1987); long-term survivors of HIV-1 have been characterized by a strong neutralizing-antibody response (Pantaleo, *et al.*, "Studies in Subjects with Long-term Nonprogressive Human Immunodeficiency Virus Infection", *N. Eng. J. Med.* 332:209-216, 1995, Cao, *et al.*, "Virologic and Immunologic Characterization of Long-Term Survivors of Human Immunodeficiency Virus Type 1 Infection", *N. Engl. J. Med.* 332:201-208, 1995); and the infusion of

D3

plasma rich in anti-HIV-1 antibody has been reported to delay the appearance of the first AIDS-defining event (Vittecoq, *et al.*, "Passive Immunotherapy in AIDS: A Double-Blind Randomized Study Based on Transfusions of Plasma Rich in Anti-Human Immunodeficiency Virus 1 Antibodies *vs*. Transfusion of Seronegative Plasma, *Proc. Natl. Acad. Sci. USA*, 92:1195-1199, 1995). $CD8^+$ T_H cells are well-recognized. Thus, there is a potential advantage to the infusion of cells that can provide T_H activity to B-cells. Some of the $CD8^+$ cells were also $CD45RA^+$ or $CD30^+$, suggesting the possibility of $CD8^+$ T_H function *in vivo*, including the induction of anti-HIV-1 antibody (Manetti, *et al.*, *supra*) The release of T_H1 cytokines, such as IFNg, suggests the possibility that DTH responses can be enhanced. The study of Carter, *et al.*, (*supra*) has suggested the feasibility and safety of infusing a mixed population of uninfected $CD4^+$ and $CD8^+$ cells into HIV-1-infected individuals.

The complexity of the cytokine system is an obstacle to effective systemic therapy. Cytokines can mediate both beneficial and detrimental effects depending on a variety of factors, including the nature of the responding cell, the stage of infection, and the concentration of the cytokine. For example, certain cytokines, including IL-4 and IL-10, enhance the replication of HIV-1 in lymphocytes but inhibit HIV-1 replication in macrophages (Akridge, *et al.*, "IL-10 is Induced During HIV-1 Infection and is Capable of Decreasing Viral Replication in Human Macrophage", *J. Immunol.* 153:5782-5789, 1994; Montaner *et al.*, "T_H2 Downregulation of Macrophage HIV-1 Replication", *Science* 267:538-539, 1995). Low concentrations of IL-4 enhance HIV-1 replication in lymphocytes, while high concentrations inhibit replication (Mackewicz, *et al.*, 1994, *supra*). Systemic therapy with intermittent courses of IL-2 has been reported to increase the number of $CD4^+$ cells; however, perhaps because CD4 cells also are activated, plasma HIV-1 RNA levels increase (Kovacs, *et al.*, "Increases in CD4 T Lymphocytes with Intermittent Courses of Interleukin-2 in Patients with Human Immunodeficiency Virus Infection--A Preliminary Study", *N. Engl. J. Med.* 332:567-575, 1995). Cytokines also are unlikely to be secreted or act individually, but rather as a "cascade" involving other cytokines, and regulated, local, *i.e.*, paracrine, release appears to be critical to optimal activity. Finally, the experience to date, including that with IL-2 therapy, suggests that systemic cytokine therapy will unlikely be of benefit in patients with advanced HIV-1 infection.

The infusion of cells as a vehicle for modulating the cytokine system addresses many of the limitations inherent to the systemic cytokine therapy. Theoretically, cells could traffic to reservoirs of HIV-1 activity and specifically release a variety of cytokines in a regulated fashion that could mediate both antiviral and immunorestorative effects. Several lines of evidence suggest a role for $CD8^+$ CTL in the control of HIV-1 infection; and, thus, the focus of most cellular immunotherapy programs has been on expanding $CD8^+$ CTL. Longitudinal studies have shown, however, that HIV-1 can

escape specific CTL recognition (Phillips, *et al.*, "Human Immunodeficiency Virus Genetic Variation that Can Escape Cytotoxic T Cell Recognition", *Nature* 354:453-459, 1991). HIV-1-specific CD8+ CTL have been demonstrated to cause immunopathology, including dementia and lymphocytic alveolitis, and may contribute to the immunodeficiency by lysing HIV-1-infected CDC4 cells (Riddell, *et al.*, *supra*; Grant, *et al.*, "Lysis of CDr+ Lymphocytes by Non-HLA-Restricted Cytotoxid T Lymphocytes from HIV-Infected Individuals", *Clin. Exp. Immunol.* 93:356-362, 1993). It also is unclear whether the severely damaged immune systems of patients with advanced disease would be capable of spontaneous regeneration even if virus replication could be completely inhibited by totally effective CD8+ CTL (Fauci, "Multifactorial Nature of Human Immunodeficiency Virus Disease: Implications for Therapy", *Science* 262:1011-1018, 1993). Other cytolytic cells, including NK cells, can mediate antiviral activity; but, whether they can reverse the immunosuppression is unknown and unlikely. It is of interest that in a number of preclinical and clinical studies targeting cancer, the effectiveness of cellular immunotherapy programs has correlated better with the ability of the infused cells to specifically secrete cytokines than with cytotoxicity *in vitro* (Barth, *et al.*, "Interferon g and Tumor Necrosis Factor have a Role in Tumor Regression Mediated by Murine CD8+ Tumor-Infiltrating Lymphocytes", *J. Exp. Med.* 173: 647-658, 1991; Kim, *et al.*, "Effects of the Adoptive Transfer of Cells Secreting Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) in Patients with Colon Cancer", *J. Immunother* 16-239, 1994; Schwartzentruber, *et al.*, "*In Vitro* Predictors of Therapeutic Response in Melanoma Patients Receiving Tumor-Infiltrating Lymphocytes and Interleukin-2", *J. Clin. Oncol.* 12:1475-1483, 1994).

Another unexpected, yet fortuitous, benefit of the present invention is the reduction in viral load that the patients experience when presented with the inventive therapeutic. Recent evidence has shown patient benefit in reducing viral loads in AIDS patients (Deeks, *et al.*, "HIV-1 Protease Inhibitors - A Review for Clinicians, *JAMA*, Vol. 277, No. 2, 145-153, January 8, 1997; Mellors, *et al*, "Quantitation of HIV-1 RNA in Plasma Predicts Outcome after Seroconversion", *Ann. Intern. Med.*, 1995; 122: 573-579; and Coombs, *et. al.*, "Association of Plasma Human Immunodeficiency Virus Type 1 RNA Level with Risk of Clinical Progression in Patients with Advanced Infection", *J. Inf. Dis.*, 1996; 174: 704-712). The data below will expand on this benefit of the present invention.

In broader terms and based on the data presented below, it is believed that the inventive cellular therapy regimen has applicability in the treatment of immune mediated diseases (of which HIV is an example) in both animals and humans. While some of these diseases are bacterial (*e.g.*, tuberculosis) and some are of unknown cause (autoimmune diseases,*e.g.*, rheumatoid arthritis), most such immune mediated diseases

are viral induced and result from persistent and acute infections, including latent infection (*e.g.*, human herpes virus), chronic infections (*e.g.*, "old dog encephalitis" following canine distemper virus (CDV) infection or lymphocyteic choriomeningitis in mice), and slow infections (both lentiviruses including HIV, feline immunodeficiency virus (FIV), and simian immunodeficiency virus (SIV); and a group of unclassified agents which cause subacute spongioform encephalopathies including Cruetzfeld-Jakob disease, Kuru, and Mad Cow Disease). Such immunosuppressive or chronic diseases that lead to an immunosuppressed state in the host (both human and animal) should be treatable in accordance with the precepts of the present invention including, for example, HIV, tuberculosis, measles, dengue fever, malaria, hepatitis (chronic), leprosy, rheumatoid arthritis, multiple sclerosis, canine distemper virus, and the like.

Chronic and acute viruses are classified as being DNA viruses or RNA viruses, enveloped and non-enveloped. RNA viruses are exemplified by, for example, picornaviruses, togaviruses, paramyxoviruses, orthomyxoviruses, rhandoviruses, reoviruses, retroviruses, bunyaviruses, coronaviruses, and arenaviruses. DNA viruses are typified by panoviruses, papoviruses, adenoviruses, herpesviruses, and poxviruses. For more information on viruses, reference is made to the following texts: Fenner, *et al.*, *Veterinarian Virology*, 2nd Edition, Academic Press, New York, New York (1993); Mims, *et al.*, *Viral Pathogenesis and Immunology*", Blackwell Scientific Publications, London, England (1984); Virology, B.N. Field, Editor, Raven Press, 3rd edition; Shulman, *et al.*, *The Biological and Clinical Basis of Infectious Diseases,* 5th edition, W.B. Saunders Co. (1997), the disclosures of which are expressly incorporated herein by reference.

The following examples illustrate the present invention, but they should not be construed as limiting. All citations referred to herein are expressly incorporated herein by reference.

EXAMPLES

EXAMPLE I
IN VITRO DATA

MATERIALS AND METHODS

Cells. Samples of lymph nodes not needed for diagnosis were obtained from resected specimens. The major part of each lymph node was used for histopathology; approximately 20% to 50% was used for the immunologic studies. Lymph nodes with histologic evidence of malignancy or mycobacterial infection were excluded. Dissociation of tissue was carried out under sterile conditions in a laminar flow hood. Tissue was rinsed in a centrifuge tube with a serum-free medium, Macrophage SFM (Gibco/BRL, Grand Island, NY), and was transferred to a Petri dish. Extraneous

tissue was excised with a scalpel, and the tissue was minced into pieces approximately 2 to 3 mm in diameter. Tissue fragments were placed in centrifugation tubes with collagenase pre-warmed to 37°C. Tubes were vortexed at a speed that caused tumbling, but not foaming. Free cells were decanted through three layers of sterile medium-wet nylon mesh into centrifuge tubes. The cells were centrifuged (250 X g) in a refrigerated centrifuge for ten minutes. The supernatant fluid was poured off, and the procedure was repeated until sufficient cells were obtained. Cells from the different digests then were pooled and counted, and the cell viability was assessed by the Trypan blue exclusion test. Cells not needed immediately were cryopreserved in dimethylsulfoxide with 5% autologous serum.

Cell Expansion. Lymph node lymphocytes (10^6/ml) were suspended in Macrophage SFM, 600 IU/ml human recombinant IL-2 (Proleukin, Cetus Oncology, Emeryville, CA), and MAb anti-CD3 (Orthoclone, OKT3, Ortho Pharmaceutical Corporation, Raritan, NY) at 37°C for four days. Cells were counted and resuspended every three to four days depending on growth in fresh media with additions.

Flow Cytometry. Cell surface marker analysis was performed on an EPICS ELITE cytofluorgraph using fluoresceinated or phycoerythrinated MAb as previously described (Triozzi, *et al.*, 1994). The following MAbs were used: anti-CD3 (Leu4), -CD4 (Leu3a), -CD8 (Leu2a), -11b (Leu15), -CD14 (LeuM3), and -CD56 (Leu19) (all from Becton-Dickinson); anti-CD30 (DAKO Corporation, Carpinteria, CA); anti-CDw60 (Pharmingen, San Diego, CA); anti-CD45RA (2H4) and -CD45RO (UCHL1; both from AMAC, Westbrook, ME); and anti-HLA-DR (HB103; ATCC, Rockville, MD).

Cytolytic Activity. Target cells were labeled with Na chromate (^{51}Cr) using 100 μCi/5 X 10^6 cells/0.5 ml for one hour at 37°C. Target cells (10^4/100 μl) were added to triplicate 6-mm round-bottomed plates. Effector cells generated, as described above, were added in the same media (in 100 μl) to achieve effector-to-target (E:T) ratios of 40:1, 20:1, 5:1, and 1.5:1. Also included were three maximum release wells containing only labeled target cells plus TRITON X-100 surface active agent and three spontaneous release wells. The plate was centrifuged (200 X g for 5 minutes) and incubated at 37°C for four hours. The plate was centrifuged again and 100 μl of supernatant was removed. The percent of lysis was determined by the formula: (experimental cpm - spontaneous cpm) / (total cpm - spontaneous cpm). Each variable was tested in triplicate.

Cytokine Production. Standard semi-quantitative polymerase chain reaction (PCR) was employed to assess cytokine mRNA expression. Total RNA was extracted by quanidium thiocyanate phenol chloroform using RNA-zol (Cinna/Biotec, Friendswood, TX). One μl of cDNA was mixed with 10 μl of 10 X PCR buffer (500 mM KCl, 200 mM Tris HCl, pH 8.3, $MgCl_2$ concentration optimized for each oligo

set), 2 µl of 20 pM of the relevant oligonucleotide, 0.5 µl of 10 mM dNTP, and 1.2 U Taq polymerase (Ampli Taq, Perkin-Elmer Cetus) to a final volume of 100 µl. Amplification was performed with a Perkin-Elmer Cetus thermocycler for 30 cycles (1 min. 94°C melting, 2 min. 55°C annealing, and 2 min. 72°C extension). To ensure that equal amounts of starting material was used, cDNA product were amplified with G3PDH primers. Amplified products were separated on a 2% agarose gel and blotted onto nylon filters. The filters were incubated in a pre-hybridization solution for four hours at 65°C and then hybridized to a digoxigen-labeled internal Cb probe (Boehringer Mannheim, Indianapolis, IN). The filters were washed twice with 2 X SSC 0.5 SDS, then in 2 X SSC 0.1 SDS before being developed with dioxigenin-specific alkaline phosphatase-coupled antibody. mRNA expression was scored as: - (negative); + (positive); and ++ (strongly positive).

Commercially available enzyme-linked immunoabsorbent assay (ELISA) kits were used to quantify IL-4, IL-5, IL-6, GM-CSF (R&D Systems, Inc., Minneapolis, MN) and IFNg, and TNFa (Genzyme Corporation, Cambridge, MA) levels. Assays were conducted according to the recommendations of the manufacturers.

<u>HIV-1 Co-Culture</u>. Quantitative HIV-1 co-cultures were performed per the ACTG Virology Manual.

<u>HIV-1 mRNA</u>. PCR was also used to quantify HIV-1 mRNA expression. mRNA was obtained and PCR performed as described above. To verify the presence of HIV-1 in a sample, oligomer SK38 and SK39 were used (Varas, *et al.*, "Influence of PCR Parameters on Amplifications of HIV-1 DNA: Establishment of Limiting Sensitivity", *Biotechniques* 11: 384-391, 1991). These oligomers amplify a 115-bp fragment of the HIV-1 *gag* gene. PCR was optimized using the SK38/SK39 primer set at the 0.8 mM dNTPS and 2.8 mM Mg^{+}.

<u>Proliferation Assay</u>. Proliferation assays were performed by incubation 10^5 lymphocytes, in both the presence and absence of IL-2, with synthetic oligopeptides corresponding to HIV-1 envelope sequence (The Ohio State University Comprehensive Cancer Center Peptide Laboratory). Cells were pulse-labeled with 1 µCi [^{3}H] thymidine for one hour at 37°C and harvested using a Skatron cell harvester.

<u>HIV-1 Suppression Assay</u>. The effects of culture supernatants on HIV-1 production were assessed using previously described methods (Mackewicz, *et al.*, 1992, *supra*). Peripheral blood $CD4^+$ T cells were isolated from a normal volunteer using negative selection (Human T cell CD4 Subset Column Kit, R&D Systems, Inc., Minneapolis, MN). These purified $CD4^+$ cells were activated with 10 ng/ml OKT3 and grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco/BRL, Grand Island, NY) and 100 IU/ml IL-2. Cells were maintained between 0.5 and 2 x 10^6/ml by adding fresh complete medium weekly. $CD4^+$ T cells (5 X 10^5/ml/well) were added to a 24-well plate containing either 20% or 80% of

supernatants from lymph node cell expansion cultures from HIV-1-infected donors in which 5, 20, or 100 ng/ml OKT3 was used. Control wells were established from cells similarly expanded from the lymph node of a cancer patient. All wells, except the "no virus" control wells, were infected with HIV-1 culture supernatant known to contain sufficient HIV-1 to infect lymphocyte cultures at the proportions used. Supernatants were collected from the 24-well plate at twice weekly intervals. At each collection, the same proportion of supernatants from the original expansion cultures was added. Day-4 and day-12 supernatants from the 24-well plate were analyzed by quantitative ELISA for HIV-1 p24 antigen (Coulter, Hiyalea, FL) as were the supernatants from the original expansion cultures. The amount of p24 produced was calculated by subtracting the p24 present in the 20% or 80% of the original expansion culture supernatant from the p24 detected in the 24-well plate. The p24 produced in control wells with fresh media alone was compared to the p24 produced in well with 20% or 80% supernatant from the original HIV-1 lymph node expansion culture or from the control cancer patient expansion culture.

RESULTS

Cell expansion. Lymph nodes were obtained from 12 HIV-1-infected donors. The number of cells obtained per lymph node specimen for immunologic studies varied from 0.3 x 10^8 to 30 x 10^8. In general, the CD4/CD8 ratio of lymph nodes paralleled that of the peripheral blood. Expansion of lymph node cells was examined in the presence of 600 IU/ml of IL-2 and a range of concentrations of anti-CD3 MAb. Initial exposure to lower concentrations of anti-CD3 MAb, namely 5 ng/ml, resulted in higher cell numbers and also favored $CD4^+$ cell expansion at 10 days (Fig. 1). After 14 days of culture, the concentration of anti-CD3 MAb did not influence overall cell yields and cells were predominantly $CD8^+$. It was determined that the concentration of IL-2 could be reduced after four days to 20 IU/ml without substantially reducing the expansion rates. Expansion was examined in a serum-free medium formulated to maintain the viability of APCs. Large cells having the morphologic appearance of macrophages were visualized in the cultures throughout. These adherent and semi-adherent cells were associated with lymphocyte colonies. Fold-expansions in serum-free conditions were equivalent to that of serum-supplemented media. There was a tendency for $CD4^+$ cell expansion to be greater in serum-free conditions. However, these differences were not statistically significant.

Culturing lymph node cells initially at 10^6/ml in serum-free media with 5 ng/ml of anti-CD3 MAb and 600 IU/ml IL-2 for the initial four days, resuspending cells at 0.25 x 10^6 cells/ml in media with 20 IU/ml IL-2 for three days, and then resuspending cells at 0.35 x 10^6 cells/ml with media and 20 IU/ml I-2 for three more days (total of 10 days) appeared to be optimal. Using this regimen, lymph node cells expanded 6.0-fold

to 48.5-fold in 10-days; expansion rates declining after 10 days. The rate of cell expansion appeared to be positively related to the number of $CD4^{+}$ cells present initially. Log-fold expansion, however, was achievable even from patients with peripheral blood CD4 counts of less than $50/mm^3$. The expansion rates of lymph nodes were greater than that of the peripheral blood cells. The percentage of CD4 and CD8 cells remained constant throughout the expansion process. Fig. 2 displays expansion of lymph node cells to peripheral blood for two representative patients.

Detailed phenotypic analysis was performed on cells from five patients using the optimal expansion regimen described above.

TABLE 1

Phenotype (% positive) of Cells Pre and Post Expansion

	Patient 1		Patient 2		Patient 3		Patient 4		Patient 5	
Phenotype	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
CD4+	5	6	21	31	15	44	19	41	32	41
CD8+	80	90	71	75	50	66	29	47	29	73
CD14+	1	0	2	0	6	0	--	--	--	--
CD56+	3	6	5	7	--	15	5	12	--	--
CD4+CD25+	8	43	--	--	--	--	--	--	--	--
CD4+CD45RA+	--	3	--	16	--	17	6	10	--	--
CD4+CD45RO+	11	64	24	57	13	--	--	40	29	38
CD4+HLA-DR+	23	84	29	90	34	91	28	95	--	--
CD8+11B+	3	2	5	2	1	2	1	0	2	3
CD8+CD30+	21	33	24	65	1	44	2	30	10	37
CD8+CD45RA+	88	85	73	69	--	--	--	--	--	--
CD8+CD45RO+	20	12	16	17	--	--	--	--	--	--
CD8+CDw60+	15	78	24	56	17	49	22	42	22	61

The majority of expanded cells were $CD8^+$. Importantly, >90% of all of the $CD8^+$ cells were $CD11b^-$, the phenotype of CTL and also $CD8^+$ cells which inhibit HIV-1 replication non-lytically (Mackewicz, *et al.*, 1992, *supra*). The expression of CD11b, the CR3 receptor, by $CD8^+$ cells has been associated with T-suppressor activity (Landay, *et al.*, "Characterization of a Phenotypically Distinct Subpopulation of Leu-2^+ Cells which Suppress T Cell Proliferative Responses", *J. Immunol.* 131:2757-2761, 1983). Approximately 3.0% - 16.9% of the $CD8^+$ cells were $CD45RA^+$, 34.0% - 65% were $CD30^+$, and 42.0% - 78.7% were $CDw60^+$, the phenotype of $CD8^+$ T_H cells (Manetti, *et al., supra*; Rieber, *et al.*, "CDw60: A Marker for Human $CD8^+$ T Helper Cells", *J. Exp. Med.* 170:1385-1390, 1994). Most of the $CD4^+$ cells that resulted expressed the activation and "memory" marker CD45RO. Very few $CD56^+$ NK cells (<5%) and no B cells (<1%) expanded. Less than 6% of the expanded expressed the monocyte/macrophage marker, CD14.

Cytokine Production. Cytokine production was evaluated by assessing cytokine mRNA expression using standard semi-quantitative PCR, measuring cytokine levels of the culture supernatants using commercially available ELISA kits, and by measuring cytokine production (ELISA) of the expanded cells exposed to immobilized anti-CD3 MAb. Expression of cytokine mRNA over the 10-day culture period is displayed in Table 2 below.

TABLE 2

Cytokine mRNA Expression

	4 Hours		3 Days		6 Days		10Days	
	IL-2	IL-2/CD3	IL-2	IL-2/CD3	IL-2	IL-2/CD3	IL-2	IL-2/CD3
IL-4	+	+	–	–	–	–	–	–
IL-5	+	++	-	+	+	++	+	++
IL-8	++	++	+	+	+	+	+	+
IL-10	+	+	+	+	+	+	+	+
IFNγ	+	++	+	+	+	+	+	+
TNFα	+	+	+	+	+	+	+	+
TFGβ	++	++	+	+	+	+	+	+
MIP-1α	+	+	+	+	+	+	+	+

The addition of MAb anti-CD3 to IL-2 resulted in increased expression of IL-5 mRNA. IL-2/anti-CD3-expanded cells consistently expressed mRNA for interferon (IFN) g, IL-1, IL-4, IL-8, tumor necrosis factor (TNF) a, and transforming growth factor (TGF) b-cytokines that have been shown to suppress HIV-1. High concentrations of GM-CSF were present in the culture supernatants (> 500 pg/ml). IL-5, IL-6, IL-10, and TNF were also present in the cell culture supernatants; secretion of these cytokines increased in response to immobilized anti-CD MAb (Fig. 3). In general, maximal secretion of cytokines in response to immobilized anti-CD3 MAb was observed from cells expanded for 8 to 10 days (Fig. 4). Note, also that MIP-1a also was expressed. The importance of this expression recently has been reported by Cocci, *et al.*, *Sci.*, vol. 270, pp 1811 *et seq.* (1995).

HIV-1 Production. Freshly collected lymph nodes grew out HIV-1; cultures of the expanded cells were negative (n = 4). HIV-1 mRNA, determined by PCR, increased after 1 to 3 days of culture, by day 6 HIV-1 mRNA it was undetectable. In contrast, HIV-1 mRNA remained positive in lymph node cells expanded in IL-2 alone (Fig. 2).

Effect of Culture Supernatants on HIV-1 Production. Culturing HIV-1-infected peripheral blood lymphocytes with immobilized anti-CD3 MAb and IL-2 has been shown previously by Levy and coworkers to induce the release of a soluble factor or factors that have been shown to inhibit HIV-1 replication in a non-major histocompatibility complex (MHC)-restricted manner (Mackewicz, *et al.*, 1994, *supra*). Whether culture conditions resulted in the production of soluble factors capable of inhibiting HIV-1 replication was evaluated by assaying the capacity of the supernatants to suppress HIV-1 replication in previously uninfected, normal $CD4^+$ cells (Mackewicz, *et al.*, 1994, *supra*). Wells with normal $CD4^+$ cells infected with HIV-1 were highly positive for the p24 antigen at both day 4 and day 12; wells not infected with virus had no p24 antigen. Supernatant from lymph node cultures from HIV-1-infected donors inhibited replication of HIV-1 in the normal $CD4^+$ cells infected with HIV-1 *in vitro*. Inhibition of HIV-1 replication was not observed at day 4 using 20% supernatant from HIV-1-infected lymph node cultures; the use of 80% supernatant, however, did result in inhibition (Fig. 6). Both 20% and 80% supernatant inhibited HIV-1 replication at day 12. HIV-1 inhibition was greater when using the supernatant form lymph node cell expanded with the highest amount, namely 100 ng/ml, of OKT3.

Proliferative Responses. To examine the spectrum of HIV-reactivity, the proliferative response of expanded cell to oligopeptides corresponding to HIV-1 envelope sequence was assessed in the presence of autologous EBV-transformed B-cells as APCs. Significant proliferative response to all epitopes was observed (Fig. 7).

Cytolytic Activity. The cytotoxic activity of the expanded cells was assessed using ^{51}Cr-release assays with a variety of target cells including autologous EBV-

transformed B-cells pulsed with HIV-associated oligopeptides. Four-day expansion demonstrated significant cytotoxicity (% specific lysis greater than 40% at effector to target ratios of 10:1) versus NK-resistant Daudi cells, *i.e.*, lymphokine activated killer (LAK) cell activity. Ten-day expanded cells were weakly cytolytic versus NK-cell sensitive Raji and NK-resistant Daudi cells (% specific lysis < 10% at effector to target ratio of 10:1). They did not specifically lyse autologous EBV-transformed B-cells that were pulsed with oligopeptides corresponding to the HIV-1 envelope sequence (% specific lysis < 10% at effector to target ratio of 10:1).

EXAMPLE II
IN VIVO DATA

1. A pilot study was conducted at The Ohio State University (Columbus, Ohio) under an IND granted by the FDA in accordance with the teachings herein.
2. Eligibility criteria in the study included: HIV-1 infection documented by ELISA and confirmed by either a clinical history compatible with HIV-1 disease, Western blot, positive HIV-1 culture, positive HIV-1 antigen, or plasma viremia; CD4 count of less then 200 but greater than 100/mm^3 within 4 weeks of the time of lymph node excision; palpable peripheral lymph nodes that were considered to be easily accessible/resectable under local anesthesia; Karnofsky performance status greater than 70; adequate organ function defined by an absolute neutrophil count greater than 1000 mm^3, hemoglobin greater than 9.5 g/dL, platelet count greater than 75,000/ mm^3, creatinine less than 1.5 mg/dl, bilirubin less than 1.5 mg/dl, and hepatic transaminases less than 3 times the upper limit of normal; and signed informed consent.
3. Exclusion criteria included: any active AIDS-related opportunistic infection, encephalopathy, or malignancy except mild Kaposi's sarcoma; significant autoimmune disease or non-AIDS-associated malignancy; prior splenectomy; prior immunotherapy; or concurrent experimental HIV-1 therapy. Patients had to be receiving approved antiretroviral treatment and *Pneumocystis carinii* pneumonia (PCP) prophylaxis, but there could not have been any change in therapy for the 4 weeks prior to enrollment.
4. Consenting, eligible patients underwent excision of a clinically palpable lymph node. The lymph node was examined for malignancy and for opportunistic infection. If histologic and microbiologic examinations were negative, lymph node cells were processed and underwent an *ex vivo* expansion. Patients received a single infusion of autologous expanded cells into a peripheral vein. They were premedicated only with 650 mg of acetaminophen. Patients were monitored frequently for clinical and laboratory evidence of toxicity. Toxicity was graded

using World Health Organization Common Toxicity Criteria. Virologic and immunologic response determinations 1 day, 2 days, 1 week, 2 weeks, and 5 weeks after cell infusion and thereafter every 4 weeks. Peripheral blood immunophenotyping was performed by The OSU Hospital Aids Clinical Trial Unit Cellular Immunology Laboratory. Viral loads were assessed by NASBA (Advanced Bioscience Laboratory, Kensington, MD) and by quantitative polymerase chain reaction (PCR; Corning Clinical Laboratories, Pittsburgh, PA; and Lab Corp., Dublin, OH). The sensitivity of these assays are 400 copies/sample (*viz.*, 100 µl or 1 ml).

5. Adequate portions of each lymph node was used for histopathology; the remainder was used for therapy. Lymph nodes with histologic evidence of malignancy or opportunistic infection were excluded. After extraneous fatty and connective tissue was removed, lymph nodes were minced into pieces approximately 2 to 3 mm in diameter under sterile conditions in a laminar flow hood. The minced lymph nodes were transferred to a 50 ml conical centrifuge tube and allowed to stand at 1 x *g* for one to two minutes in a serum-free medium (Macrophage SFM, Gibco BRL, Grand Island, NY) to allow dense capsular material to settle. Cells and supernatant were pipetted into a fresh 50 ml tube. The cells were then centrifuged (250 x *g*) at room temperature for six minutes. The supernatant and any fatty accumulation on the surface were carefully aspirated and discarded. The cell pellet was resuspended in fresh serum-free medium. An aliquot of cells was counted and viability assessed by Trypan blue exclusion.

6. Lymph node cells were cultured in serum-free medium at a density of 10^6/ml with 10 ng/ml of anti-CD-3 monoclonal antibody (MAb) (Orthoclone, OKT3, Ortho Pharmaceutical Corporation, Raritan, NY) and recombinant human IL-2 (Proleukin, Cetus Oncology Corporation, Emeryville, CA) at 600 IU/ml. Cultures were maintained in air-porous plastic bags (Ethox, Buffalo, NY) in 5% CO_2 in humidified air at 37°C. Cells were resuspended after 3 to 4 days, depending on growth, at 0.25 x 10^6/ml in fresh medium containing IL-2 at 600 IU/ml, and then resuspended after 3 to 4 more days at a density of 0.35 x 10^6/ml in fresh medium containing IL-2 at 120 IU/ml. Cells were harvested and washed in 1 L of 0.9% NaCl and 1.25% human serum albumin (American Red Cross) using a SteriCell harvester (E.I. DuPont de Nemours and Co., Glenolden, PA) and suspended in 300 ml of 0.9% NaCl, 1.25% normal human serum albumin in a transfer bag. The following were performed to characterize the cells prior to infusion: cell number and viability, endotoxin assay of final product supernatant, morphology of cytocentrifuge preparations stained with Diff Quik (Baxter Healthcare Corp., Miami, FL); gram stain; cultures for bacterial contamination; and HIV-1 mRNA.

7. RT-PCR was used to assess an HIV-1 expression. PCR primers were designed using primer analysis software (Oligo, National Biosciences, Inc., Plymouth, MN) and obtained from Stratagene (La Jolla, CA). The HIV-1 primer corresponded to the *gag* gene (SEQ ID NO: 1) (5'-primer = ATAATCCACCTATCCCAGTAGGAGAAAT; (SEQ ID NO: 2) 3'-primer = TTTGGTCCTTGTCTTATGTCCAGAATGC; PCR product = 873 bp). Total RNA was extracted via guanidine isothiocyanate phenol using TRIzol (Gibco/BRL, Gaithersburg, MD). Equal starting concentrations of total RNA (0.5 µg) were used as a template for the RT reaction. RT synthesized cDNA (2 µL) was mixed with 1.8 µL of 10X PCT buffer (500 mM KCl, 200 mM Tris HCl, pH 8.3), 1.625 mM $MgCl_2$, 1 µL of the relevant 10 µM oligonucleotide primer set, and 0.5 U Taq Polymerase (Gibco/BRL) to a final volume of 20 µL. RNA from HIV-1-infected and uninfected cells were used as controls. GADPH cDNA was amplified for each sample to serve as an internal control (SEQ ID NO: 3) (5'-primer = CTACTGGCGCTGCCAAGGCTGT; (SEQ ID NO: 4) 3'-primer = GCCATGAGGTCCACCACCCTGT; PCR product = 358). Amplification was performed with a Perkin-Elmer Cetus thermocycler for 40 cycles of template denaturing (30 seconds at 94°C), annealing and extension (2 minute at 60°C). Amplified products were separated on a 1% agarose gel and visualized using a UV illuminator.

8. Cell surface marker analysis was performed on an EPICS ELITE cytofluorograph using fluoresceinated and phycoerythrinated MAb as previously described (Triozzi, *et al.*, "Identification of tumor-reacting lymph node lymphocytes *in vivo* using radiolabeled monoclonal antibody", *Cancer*, 73:580-589, 1994). The following MAbs were used: anti-CD3 (LEU4), -CD4 (LEU3a), -CD8 (Leu2a), -11b (Leu15), -CD14 (LeuM3), and -CD56 (Leu19) (all from Becton-Dickinson); anti-CD30 (DAKO Corporation, Carpinteria, CA); anti-CDw60 (PharMingen, San Diego, CA); anti-CD45RA (2H4) and -CD45RO (UCHL1; both from AMAC, Westbrook, ME); and anti-HLA-DR (HB103; ATCC, Rockville, MD).

9. Expanded cells were stimulated with irradiated (75 Gy), autologous, Epstein-Barr virus (EBV)-transformed B-cell lines transfected with either empty *Vaccinia* control vector or the *Vaccinia* vPE16 vector, which contains the HIV-1 *env* gene. The EBV-transformed cell lines were generated from peripheral blood lymphocytes using B95-8 marmoset cell line supernatant by standard methods (Blumberg, *et al.*, "Effects of human immunodeficiency virus on the cellular immune response to Epstein-Barr virus in homosexual men: characterization of the cytotoxic response and lymphokine production, *J. Infect. Dis.*, 155:877-890 1987). The vectors were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Health (*Vaccinia*

control, Dr. Bernard Moss; vPE16, from Drs. Patricia Earl and Bernard Moss). Expanded cells were cultured for four additional days with the stimulator cells at a ratio of 10:1, and then supernatants were collected. ELISA kits were used to quantify MIP-1a and RANTES levels (R&D Systems Inc., Minneapolis, MN). Assays were conducted in duplicate according to the recommendations of the manufacturer, and data are presented as mean values of these determinations.

10. Cells were cultured at 10:1 with irradiated, autologous EBV-transformed B-cell lines transfected with either empty *Vaccinia* control vector or *Vaccinia* vPE16 vector as described above. After four additional days, the cells were pulsed with ^{3}H-thymidine for four hours, harvested, and counted. Data are presented as mean cpm ± SD for quadruplicate determinations.
11. Cytolytic activity versus the autologous B-cells expressing *env* protein, natural killer (NK) sensitive Raji cells, and NK-resistant Daudi cells was assessed at an effector-to-target ratio of 10:1 for four hours, as previously described (Triozzi, *et al.*, *supra*). Also included were three maximum release wells containing only labeled target cells plus TRITON X-100 surfactant and three spontaneous release wells (no effector cells). The percent of lysis was determined by the formula: (experimental cpm - spontaneous cpm)/(total cpm - spontaneous cpm). Each variable was tested in triplicate; data are presented as mean ± SD. Expanded/activated lymph node lymphocytes from HIV-1 infected donors did not exhibit any specific cytolytic activity against autologous B cell targets expressing the HIV-1 envelope protein gp120 (% specific lysis was less than 10% at an effector to target ratio of 10:1.
12. Skin testing for a delayed type hypersensitivity (DTH) response to common recall antigens was performed prior to and four weeks after cell infusion using the Multitest CMI skin test reaction file (Connaught Laboratories, Swiftwater, PA). Forty-eight hours after application, the largest perpendicular diameters were measured according to the recommendation of the manufacturers. Data are presented as the sum of the means of these determinations. A re-establishment of sensitivity to such antigens would establish immunorestoration of the patients.

Lymph nodes were resected from five patients. Characteristics of these patients are displayed in Table 3 below.

TABLE 3

Patient Characteristics

No.	Age/Sex	Clinical History	Antiviral Therapy	CD4 Count (Cells/ul)
1	42/M	hairy leukoplakia, thrush, chronic diarrhea, recurrent sinusitis and bronchitis, genital warts	AZT, 3TC	143
2	25/M	*Giardia*, recurrent sinusitis and bronchitis, herpes zoster, thrush	DDI, Norvir	165
3	29/M	PCP, viral meningitis, thrush	3TC	150
4	38/M		3TC	119
5	51/M	thrush, PCP, herpes zoster	3TC, D4T invirase	141

Substantially fewer cells were obtained from two resections (Patients Nos. 3 and 4). On pathologic examination, these two lymph nodes were characterized by lymphocyte depletion and dense plasma cell and eosinophil infiltration replacing most of the normal lymph-node architecture. The three lymph nodes that yield more than 10^8 cells were characterized histologically by follicular and interfollicular hyperplasia. All lymph nodes were negative for malignancy and evidence of microorganisms, including acid fast bacilli and fungus.

Cells were expanded *ex vivo* for 10 to 14 days. Characteristics of the cells, which expanded 2.3 to 200-fold, are displayed in Table 4 below.

TABLE 4

Cell Characteristics

Patient No.	Cell Number (10^8)		Cell Surface Phenotype (% Positive)				
			CD4+		CD8+		
	Initial	Final		CD45RO+[a]		CD11b+[b]	CDw60+[b]
1	3.7	46	15	84	90	9.7	70.7
2	5.1	12	27.2	96	77	17.2	44.4
3	0.24	56	ND	ND	ND	ND	ND
4	0.22	28	ND	ND	ND	ND	ND
5	5.7	26	3.3	67	86	13.1	26.2

[a] Percent CD4 cell also positive for CD45RO.
[b] Percent CD8 cell also positive for CD11b or CDw60.

The expanded cells were predominantly CD8$^+$ (Table 4). Less than 3% of the expanded cells were CD14, CD19, or CD16 positive. When challenged and transfected autologous B-cell targets expressing HIV-1 *env* protein, the expanded cells proliferated and secreted MIP-1a and RANTES. Expanded cells were weakly cytolytic versus NK-cell sensitive Raji (data not shown), NK-resistant Daudi cells, and the autologous EBV-transformed B-cells expressing the *env* gene (Table 4). Expression of HIV-1 mRNA was negative by cells expanded from Patients Nos. 1, 2, and 5; it was positive by cells expanded from Patents Nos. 3 and 4. Because of the persistent positivity, Patents Nos. 3 and 4 did not receive cell infusions and were excluded from the study.

Patients Nos. 1, 2, and 5 received a single cell infusion. The cell infusion was well tolerated with only mild (grade 1) arthralgia reported by one patient. No acute or chronic toxicities were observed. Importantly, the increases in viral load that occurred the day after cell infusion (see below) were not associated with any clinical symptoms. Infectious complications, opportunistic or otherwise, have not been observed for up to 25 weeks after infusion.

Viral loads were performed by certified clinical laboratories using NASBA (copies/100 μL) or quantitative PCR methodologies (copies/mL, where indicated). Tables 5A-C displays the changes in viral load after infusion. As expected, viral load was observed to increase the day after cell infusion. Viral load was observed to decrease to undetectable levels (<400 copies/sample being the detection limit) four weeks after cell infusion.

Tables 5A-C display the changes in peripheral blood CD4 and CD8 lymphocyte counts. Significant changes attributable to cell infusion were/were not observed. Patient No. 2 manifested sustained increases in CD4 count that may have been due, in part, to better compliance with anti-retroviral therapy. As an assessment of immunocompetence, DTH skin test reactivity to common recall microbial antigens was evaluated. All patients were poorly reactive prior to cell infusion; skin test reactivity increased after cell infusion (see Tables 5 and 6, below).

TABLE 5A

Patient 1 Results

Week #	Viral Load (copies/100 µl)	CD4		CD8		CD3		CD4:CD8 Ratio
		#/µl	% CD4	#/µl	% CD8	#/µl	% CD3	
0*	10,000	143	16	500	56	679	76	0.29
Day 2	520,000	144	17	491	58	660	78	0.29
1	22,000	--	--	--	--	--	--	--
2	21,000	--	--	--	--	--	--	--
5	< 400	125	18	362	52	501	72	0.35
9	< 400	--	--	--	--	--	--	--
13	980	171	19	504	56	693	77	0.34
17	3,600	126	--	393	53	--	--	0.32
21	2,100	211	22	528	55	749	78	0.40
25	1,000	207	20	590	57	818	79	0.35

* CD4, etc. counts are enrollment numbers 3 days prior to surgery.

TABLE 5B

Patient 2 Results

Week #	Viral Load (copies/100 µl)	CD4 #/µl	CD4 % CD4	CD8 #/µl	CD8 % CD8	CD3 #/µl	CD3 % CD3	CD4:CD8 Ratio
0	< 400*	165	15	517	47	--	--	0.32
Day 1	< 400	461	23	1,023	51	1,605	80	0.45
Day 2	< 400	419	21	998	50	1,496	75	0.42
1	< 400	431	23	881	47	1,388	74	0.49
5	--	489	23	998	47	1,614	76	0.49
9	< 400	370	24	755	49	1,201	78	0.49
14	< 400**	520	23	1.086	48	1,719	76	0.48
18	< 400	534	24	1.113	50	1.714	77	0.48
21	< 400	542	28	832	43	1,432	74	0.65
25	< 400	626	27	1,066	46	1,784	77	0.59

* 2,000 copies/ml by PCR 4 days after surgery.
** copies/ml by PCR.

TABLE 5C

Patient 5 Results

Week #	Viral Load (copies/100 µl)	CD4		CD8		CD3		CD4:CD8 Ratio
		#/µl	% CD4	#/µl	% CD8	#/µl	% CD3	
Enroll	311,070*	141	12.9	382	34.8	561	51	0.37
0	87,670**	--	--	--	--	--	--	--
Day 1	78,000	122	5	1,145	47	1,413	58	0.11
Day 2	--	198	7	1,503	53	1.899	67	0.13
1	80,000	132	7	830	44	1.038	55	0.16
2	130,000	--	--	--	--	--	--	--
5	63,000	140	8	805	46	1.049	60	0.17
9	15,000	86	6	543	38	728	51	0.16
13	1,100	--	--	--	--	--	--	--

* Copies/ml by PCR about 1 month prior to surgery.

TABLE 6

Skin Test Reaction

No.*	Tetanus		Diphtheria		Streptococcus		Tuberculin		Candida		Trichophyton		Proteus	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	7	9	0	0	0	0	0	0	3	3	0	3	0	4
2	0	7.5	0	0	0	0	0	0	3	2.5	0	3.5	0	
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Patients 3 and 4 were not treated.

The results of this study indicate that a single infusion of autologous, noncytolytic, chemokine-producing, lymph node lymphocytes is safe and can mediate beneficial virologic and immunologic activity, even in patients with advanced disease. There were no adverse effects associated with the cell infusion. After a transient increase, HIV-1 viral load was observed to decrease to undetectable levels.

Importantly, there was evidence of an improvement in immunologic function, as skin test reactivity to recall microbial antigens improved. Without the treatment described herein, negative skin test results would remain negative. "Immunorestoration" or "immunorestorative effect", as described herein, relates to the restoration of immune function (as measured by skin tests to antigens) which results by dint of the therapeutic administration of expanded lymph node lymphocytes as disclosed herein.

Technically, the procedure is easy, expedient, and reproducible. The technique employed as disclosed herein was not designed to maximize cytolytic activity, as cells were exposed to decreasing concentrations of IL-2 in serum-free media.

EXAMPLE III

MITOGENIC STIMULATION DATA

In order to demonstrate the use of different mitogens in the stimulation of the lymph node lymphocytes, lymph node lymphocytes from an HIV+ donor were stimulated with three different mitogenic stimuli: (1) anti-CD3 and IL-2 (control); (2) anti-CD3, anti-CD28, and IL-2; and (3) phytohemagglutinin (PHA) and IL-2. Anti-CD3 was used at 10 ng/ml, anti-CD28 was used at 10 ng/ml, and PHA was used at 0.5 μg/ml. Instead of the usual 100 Cetus units/ml, only 20 Cetus units/ml was used throughout the culture period. All cultures were split 1:5 on day 5 of the culture (approximately 0.25×10^6 cells/ml after split), then split to 0.35×10^6 cells/ml on day 8 of culture, and then were harvested on day 11 of culture.

All 3 cultures proliferated in response to the mitogenic stimuli used, and all 3 cultures showed functional activity against HIV in that they cleared HIV from the cultures as determined by RT-PCR performed on day 11, as demonstrated in Table 7 below.

TABLE 7

Mitogenic Stimulus	Fold Expansion		HIV RT-PCT
	After 8 Days	After 11 Days	
Anti-CD3, IL-2	3.6	9.05	Negative
Anti-CD3, Anti-CD28, IL-2	10.0	25.14	Negative
PHA, IL-2	8.2	17.34	Negative

Phenotypically, all 3 cultures were similar. Also, all 3 cultures were 98%-99% CD3+ (T cells) and displayed other surface markers in comparable quantities as demonstrated in Table 8, below.

TABLE 8

Phenotypic Marker(2)	Mitogenic Stimulus(1)		
	Anti-CD3, IL-2	Anti-CD3, Anti-CD28, IL-2	PHA, IL-2
CD3	97.8	99.6	98.3
CD44	84.4	94.3	80.3
CD3+CD44	79.8	91.9	76.0
CD4	59.6	42.9	54.7
CD45RO	92.5	89.9	78.4
CD4+CD45RO	57.8	40.2	43.9
CD8	40.5	56.6	42.5
CD11b	45.3	26.4	15.0
CD8+CD11b	5.9	4.3	2.1
CD56	2.9	2.2	2.8

(1) Numbers represent the percentage positive cells for the designated marker(s).
(2) Brief explanation of phenotypic markers:

CD3 = T cells
CD44 = HERMES antigen, lymphocyte homing receptor
CD4 = Class II-restricted T cells (helper cells)
CD45RO = activation marker, "memory" T cells
CD8 = Class I-restricted T cells (cytotoxic/suppressor cells)
CD11b = Mac-1 adhesion molecule
CD8+CD11b = cytotoxic T cell
CD56 = Natural Killer cell (LAK, Lymphokine activated killer cell)

Taken together, these data indicate that equivalent stimulation results from using any of these mitogenic regimens. Equivalent results, thus, would be expected when using any of these stimulated cells in HIV+ patients as described in Example II, above.

EXAMPLE IV

Culture Supernatants from Human Lymph Nodes Inhibit the *in vitro* Replication of Human Rhinoviruses

A. Experimental design:

Cultured supernatants from human lymph nodes were tested for antiviral activity against a DNA virus (herpes simplex virus type 1, HSV-1) and an RNA virus (human rhinovirus type 13, HRV-13). Host cells for antiviral studies were grown in 12-well tissue culture plates for 3 days before being used. After 3 days of cellular growth, mature monolayers were treated for 1-1/2 hours with either experimental material (lymph node supernatants) or control material (cell culture medium). The experimental material was used full strength (neat). After treatment of cell monolayers for 1-1/2 hours, viruses (HSV-1 or HRV-13) were added to monolayers and each virus was allowed to adsorb for 2 hours. Each virus was used at a low input of infection so individual viral plaques could be detected for quantitation in regards to plaque number and size. Following viral adsorption, all viral inoculums were removed from the monolayers and a 0.6% agarose overlay was added to each monolayer. Incorporated into the agarose overlay was either experimental material at a concentration of 80% (V/V) or control material at a concentration of 80% (V/V). After adding the agarose overlay medium, cultures were incubated for 5 days at the appropriate temperature (36.5° C for HSV-1 and 32° C for HRV-13). After 5 days of incubation, cultures were fixed and stained for the quantitation of plaque numbers and plaque size.

B. Results:

Human rhinovirus type 13 was inhibited 50% by the experimental material when compared to the control material. In addition, plaque size was also reduced by more than 50% by the experimental material. Human HSV-1 was not inhibited by the test material (plaque numbers and plaque sizes were equivalent for control and experimental samples).

C. Conclusions:

Human rhinovirus type 13 was dramatically inhibited by human lymph node supernatant fluids. Inhibition occurred in regard to a reduction in plaque numbers and plaque size. Because of the experimental design and results (reduced plaque size, *etc.*), the data suggest that the antiviral activity in the experimental material was not due to a direct virocidal effect on the virus. Instead the antiviral activity appears to be targeted at some key viral function (s) involved with replication events within host cells.

D. Specificity and controls for antiviral activity:

The experimental material was tested against 2 different viruses, an enveloped virus (HSV-1) and a nonenveloped virus (HRV-13). Both viruses were tested at the same time. In addition, the same test conditions were used for each virus. Only one virus was inhibited, the nonenveloped RNA virus. These results argue in support of an antiviral effect against a particular type of RNA virus. Appropriate controls for cell growth, pipetting, inoculum volumes, incubation conditions, and temperatures were included and should not be a factor in the results obtained. These data result from initial testing of the invention with other viruses than HIV. It is believed that these results would improve upon further testing and evaluation of the present invention and, thus, represent the potential that the present invention opens up in the treatment of immune mediated diseases in general and specifically for chronic viruses.

EXAMPLE V

Culture Supernatants from Human Lymph Nodes Inhibit the *in vitro* Replication of Herpes Simplex Virus (HSV-1) and Human Adenovirus Type 2 (Ad-2)

HSV-1 Virus

Herpes Simplex Virus Type 1 (HSV-1)
Strain: F(1), ATCC VR-733 (American Type Culture Collection)
Negative Control: EMEM with 2% fetal bovine serum (FBS)
Test System: Vero monkey kidney cells

Ad-2 Virus

Human Adenovirus Type 2 (Ad-2)
Strain: Adenoid 6 (American Type Culture Collection)
Negative Control: EMEM with 2% fetal bovine serum (FBS)
Test System: A549 cells

The appropriate cells were seeded at 150 μl per well, prior to the day of inoculation. On the day of inoculation, the media was aspirated from each well. The stock virus was serially diluted in ten-fold steps in EMEM with 2% FBS. Each dilution of the virus was inoculated into 32 replicate wells, at 50 μl/well. The viral inoculum was adsorbed for 70 ± 10 minutes at 36 ± 2°C. After adsorption, the four replicates of each virus dilution were re-fed with an individual cell supernatant or medium control dilution. The cultures were incubated at 36 ± 2°C until the final CPE observation on day 14 for HSV-1 and on day 17 for Ad-2.

TABLE 9
HSV-1 Virus
Supernatant D (80%)

Log_{10} Dilution	Number of CPE Positive Wells	Total No. of Wells
7.0	0	4
8.0	0	4
9.0	0	4
10.0	0	4
11.0	0	4
	Log_{10} TCID 50/ml	≤ 8.13*

* Theoretical titer.
Note: Cytotoxicity was observed; however, CPE was evaluated

TABLE 10
HSV-1 Virus
Medium Control (80%)

Log_{10} Dilution	Number of CPE Positive Wells	Total No. of Wells
7.0	2	4
8.0	0	4
9.0	0	4
10.0	0	4
11.0	0	4
	Log_{10} TCID 50/ml	8.30

Supernatants and controls titers were within 1 log of the certified titer. The lowest dilution of virus tested in 80% Supernatant D tested had no CPE present for the HSV-1 samples. CPE was observed in 2 of the 4 wells at the lowest dilution of virus tested in control medium. Thus, the noted Supernatant D was able to suppress or reduce virus infectivity as exemplified by HSV-1. While these tests were limited by available material, they do show the potential for the lymphocyte proliferation technique disclosed herein to be useful in treating chronic viral infections. Note, that these tests relied on the cell supernatants to achieve virus suppression as a means of screening viruses.

TABLE 11
Ad-2 Virus
Supernatant C (20%)

Log_{10} Dilution	Number of CPE Positive Wells	Total No. of Wells
7.0	3	4
8.0	0	4
9.0	0	4
10.0	0	4
11.0	0	4
	Log_{10} TCID 50/ml	8.60

TABLE 12
Ad-2 Virus
Medium Control (20%)

Log_{10} Dilution	Number of CPE Positive Wells	Total No. of Wells
7.0	4	4
8.0	3	4
9.0	0	4
10.0	0	4
11.0	0	4
	Log_{10} TCID 50/ml	9.55

Supernatants and controls titers were within 1 log of the certified titer. The lowest dilution of virus tested in 20% Supernatant C tested had CPE present in 3 of the 4 wells at the lowest dilution for the Ad-2 samples. CPE was observed in 4 of the 4 wells at the lowest dilution of virus tested in control medium. Thus, the noted Supernatant C was able to suppress or reduce virus infectivity as exemplified by Ad-2. While these tests were limited by available material, they do show the potential for the lymphocyte proliferation technique disclosed herein to be useful in acute viral infections. Note, that these tests relied on the cell supernatants to achieve virus suppression as a means of screening viruses.